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Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system

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Abstract

Parameters that affect cellular transfection as accomplished by introducing DNA via carriers composed of cationic synthetic amphiphiles, have been investigated, with the aim to obtain insight into the mechanism of DNA translocation. Such insight may be exploited in optimizing carrier properties of synthetic amphiphiles for molecules other than nucleic acids. In the present work, the interaction of vesicles composed of the cationic amphiphile dioleyloxy-propyl-trimethylammonium chloride (DOTMA) with cultured cells was examined. The results show that optimal transfection is dependent on the concentration of lipid, which determines the efficiency of vesicle interaction with the target cell membrane, as well as the toxicity of the amphiphiles towards the cell. A low lipid/DNA ratio prevents the complex from interacting with the cell surface, whereas at a relatively high amphiphile concentration the complex becomes toxic. Translocation efficiency is independent of the initial vesicle size but is affected by the size of the DNA. An incubation time of the DNA/amphiphile complex and cells of approx. 2–4 h is required for obtaining efficient transfection. In conjunction with observations on DNA/amphiphile complex-induced hemolysis of erythrocytes, a mechanism of DNA-entry is proposed which involves translocation of the nucleic acids through pores across the membranes rather than delivery via fusion or endocytosis. Dioleoylphosphatidylethanolamine, a phospholipid frequently used in a mixture with DOTMA ('lipofectin') strongly facilitates this pore formation. Translocation of the DNA is effectively prevented when the cells are pretreated with Ca^{2+} or pronase. These observations suggest that Ca^{2+}-sensitive cell surface proteins play a role in amphiphile-mediated DNA translocation.

Keywords: Transfection; DOTMA; COS-7; Carrier; Synthetic amphiphile

1. Introduction

To cure genetic disorders, therapies need to be developed that repair the genetic defect rather than modulate symptoms. Obviously, gene therapy will play a major role in future treatment. However, in order for gene therapy to be effective, it is necessary to find methods and vectors to transport the degradation-sensitive DNA to the cells and introduce this DNA into these cells.

Vesicles composed of synthetic amphiphiles, i.e., molecules with a distinct hydrophilic and hydrophobic part, which can form bilayer structures when suspended in an aqueous environment, have been shown to be effective carriers for introducing DNA into several cell lines [1–4]. Vesicles prepared from DOTMA/DOPE (1:1), also known as lipofectin [4], are widely used for accomplishing transfection [3–6] although much effort is invested in the synthesis of other, less toxic amphiphiles able to bind and transport the DNA [7,8] in an even more efficient manner. To develop novel carriers based on synthetic amphiphiles it is crucial to clarify the mechanism by which vesicles prepared from such molecules express their transfection potential. Evidently, the positive charges on the DOTMA molecules are important for the binding of the negatively charged DNA [9]. It has also been shown that DOTMA-induced transfection is greatly facilitated when DOPE, an in isolation hexagonal-phase forming amphiphile, is included in the vesicle bilayer [10].

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Although much attention has been focussed on elucidating the mechanism by which the DNA is introduced into the cells, a common and generally accepted mechanism has not been identified. Some authors tend to believe that fusion events are involved in the uptake of DNA [4,11,12], but others suggest that intracellular delivery of the DNA occurs via endocytosis [3,13].

In this study several transfection-modulating parameters were examined in order to extend the insight into the mechanism by which vesicles of synthetic amphiphiles act as carrier systems. In addition, structural features of the complex formed between nucleic acids and amphiphiles were analyzed in some detail. It is thought that these studies may govern innovative developments in the synthesis and application of novel, more effective, non-toxic amphiphiles, also capable of introducing molecules other than nucleic acids into cells.

2. Materials and methods

2.1. Cell culture

The COS-7 cell line is a derivative of the simian kidney cell line CV-1, transformed with a mutant of the simian virus 40 [14]. The cells were cultured in Costar flasks in Dulbecco's modified Eagle's medium (DMEM, Gibco, The Netherlands), containing 7% fetal calf serum (FCS), 2 mM L-glutamine (Gibco, The Netherlands), 100 units/ml penicillin (Gist-Brocades, The Netherlands) and 100 μg/ml streptomycin (Biochemie, Austria) at 37°C in CO_2/air (1:19).

2.2. Preparation of vesicles

A solution of *N*-(1-(2,3,-dioleyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE, Avanti Polar Lipids, USA) in chloroform was dried under a stream of nitrogen. The residual solvent was removed under vacuum. The lipid film was then redissolved in water and sonicated to clarity in a bath sonicator in a closed vial. The small unilamellar vesicles thus obtained were sterilized by filtration. The size of the vesicles was measured using a particle size analyzer (Nicomp, model 370). DOTMA was synthesized following a procedure as described by Felgner et al. [4], modified as outlined in Fig. 1. To quaternize the ammonium headgroup methyl bromide was used in acetonitrile. The amphiphatic tails were added as follows. NaH was washed with n-pentane, DMSO was added and the solution was stirred for 15 min. Subsequently, the product of the first reaction was added and stirred for 30 min at 30°C, after which the mixture was cooled down to 20°C. Oleyl triflate was added and the solution was heated for 2 h at 50°C. The product was purified on silica gel. The counterion was replaced by Cl^- on an ion-exchange column.

2.3. Preparation of DNA-constructs

The pCAT control vector (pCAT, Promega, USA) was incubated for 2 h at 37°C with the restriction enzyme AatII. Fragments of phage λ-DNA of known length were ligated with the plasmid DNA. To this end, plasmid DNA was mixed with the λ-DNA fragments in a ratio of 1:3. After incubation for 5 min at 50°C, the mixture was put on ice and 1 mM ATP and 0.2 U/μl T4 DNA ligase were added in a final volume of 22 μl. Ligation was allowed to proceed for 2 h at room temperature (RT) or overnight at 4°C. To prevent the pCAT from self-ligation, the plasmid was treated with alkaline phosphatase, which removes the terminal phosphate groups [15].

2.4. Transfection assay

The cells were transfected by adding a complex of DNA (pCAT) and vesicles (DOTMA/DOPE = 1:1), prepared by mixing lipid and nucleic acid at appropriate ratios, as described in the legends. The CAT activity was determined as follows. Two days after transfection, cells were lysed with 300 μl reporter lysis buffer (Promega, USA). 100 μl of lysed cells were incubated with 17 μl 0.25 M Tris-HCl, pH 7.4, 5 μl 5.5 mM n-butyryl CoA (Fluka) and 3 μl D-threo-[*dichloroacetyl*-1-^{14}C]chloramphenicol (specific activity = 56 mCi/mmol) (Amersham) for 90 min at 37°C. After the incubation, the reaction was stopped by adding 300 μl of mixed xylenes (Aldrich). The samples were vortexed for 30 s and centrifuged for 3 min at RT in an Eppendorf table centrifuge at maximum speed. The organic phase was extracted with 100 μl 0.25 M Tris-HCl, vortexed and centrifuged again. 200 μl of the organic phase was put into a scintillation vial and assayed for radioactivity.

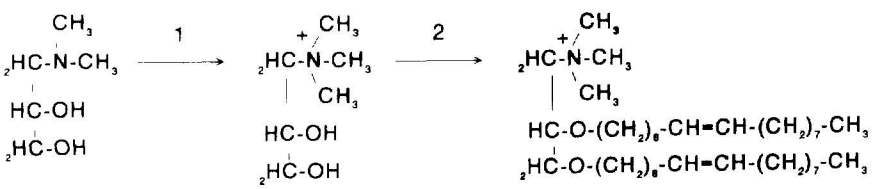

Fig. 1. Synthesis of DOTMA. Key: 1 = CH_3Br/CH_3CN, 2 = NaH/DMSO/oleyltriflate.

2.5. Measurement of EtBr-fluorescence

For EtBr-fluorescence measurements, complexes of DOTMA/DOPE and DNA were prepared as described in the legends. The complexes were incubated at RT for 10 min after which ethidium bromide (EtBr) was added to a final concentration of 0.2 μg/ml. The fluorescence was measured using a luminescence spectrophotometer AB2. The $\lambda_{excitation}$ was set at 290 nm and the $\lambda_{emission}$ was set at 600 nm.

2.6. Hemolysis of erythrocytes

Erythrocytes were isolated from whole blood samples. 10 ml of A^+ red blood cells were added to 40 ml KPN buffer solution (120 mM KCl, 10 mM $Na_2HPO_4 \cdot 2H_2O$, 30 mM NaCl, pH 7.4). The cells were centrifuged at 5°C (5 min at 3000 rpm) and the pellet was resuspended in 40 ml KPN-buffer. Cells were recentrifuged until the supernatant was clear. The pellet was then resuspended in 30 ml KPN-buffer.

10^8 erythrocytes were added to the DNA/vesicle complex in a total volume of 0.6 ml. After various time intervals at 37°C, 0.4 ml Hepes-buffered saline (HBS) was added and the cells were centrifuged immediately (3 min at maximal speed in an Eppendorf table centrifuge at 5°C). The supernatant was then screened for the presence of hemoglobine by measuring the absorbance at 540 nm. To determine the 100% value, the cells were lysed with Triton X-100 (1% v/v, final concentration).

3. Results

3.1. Parameters affecting transfection capacity

To optimize the transfection protocol, different parameters were screened for their influence on transfection. First of all, the dependence on cell type was examined. Relative to the transfection efficiency of COS-7 cells and CV-1 cells, which are known to be readily transfectable [4,14], a less efficient transfection was obtained for AD2780 cells (approx. 70%). Baby hamster kidney (BHK) cells and human fibroblasts were entirely resistant to transfection with lipofectin. Hence, in accordance with data reported before [4], transfection is clearly dependent on the cell type used.

The transfection efficiency was furthermore affected by the concentration of amphiphile as shown in Fig. 2A. When the amount of amphiphile increases, the transfection efficiency increases. Above a concentration of approx. 35 μM, the transfection efficiency decreases again. Concomitantly, the total amount of viable cells decreases at those conditions, as measured by protein content [16]. This observation indicates that when the concentration of the amphiphiles exceeds a critical value, the surfactant

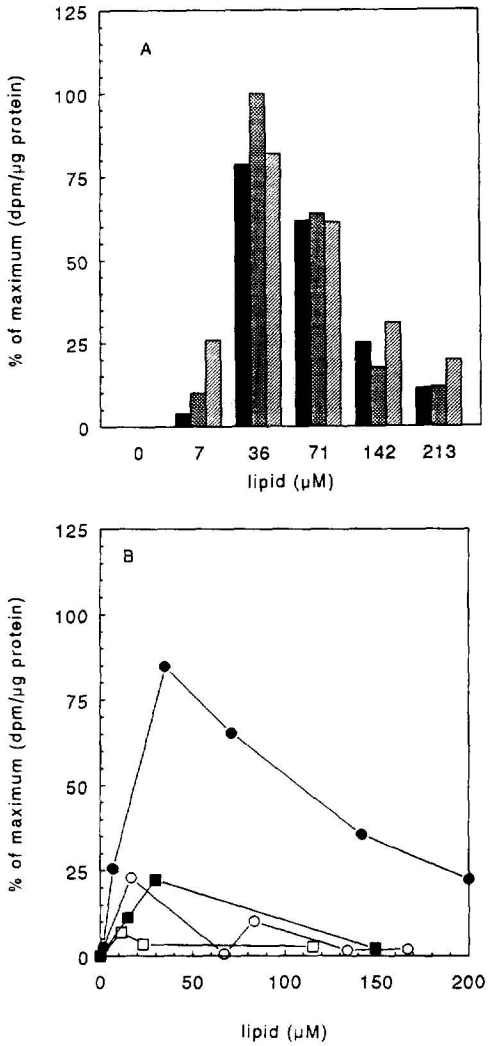

Fig. 2. Transfection of COS-7 cells as a function of vesicle concentration, vesicle size and DNA length. The cells were transfected as described in Section 2, except for the variations mentioned below. Cells were harvested after 2 days and CAT activity was determined and expressed as the amount of radioactivity per μg of cell protein. (A) Various sizes of vesicles were used in parallel transfections. The different amounts of vesicles (as indicated in the figure) were complexed to 1 μg pCAT of 4.7 kB. SUV of 80 nm (filled bar), LUV of 200 nm (double cross hatched bar) or LUV of 400 nm (hatched bar) were used. (B) The length of the DNA was varied. The cells were either transfected with a complex of vesicles and 1 μg DNA of 4.7 kb (filled circle), with 1 μg DNA of 6.5 kb (filled square) with 1 μg DNA of 8.3 kb (open square), or with 1 μg DNA of 18.8 kb (open circle).

molecules become toxic for the cells, thus providing support to results reported previously [4,20,21]. Next we examined whether complexes prepared by varying the size of the vesicles or by varying the length of the plasmid DNA, affected the transfection. As a function of DNA length, the transfection efficiency decreased with increasing DNA length (Fig. 2B). Evidently, DNA with a length of 4.7 kb led to the most efficient transfection. Note that it is reasonable to assume that the transcription efficiency of each plasmid, sharing identical promoters, is the same. As

shown in Fig. 2A the vesicle size, which varied from 80 nm to 400 nm, had no significant influence on the transfection efficiency, i.e., the same transfection pattern could be observed for all vesicle sizes investigated.

3.2. Amphiphile / DNA complexes induce hemolysis

Addition of the vesicle/DNA complex to erythrocytes caused release of hemoglobine. Hemolysis was dependent on the concentration of lipid and increased when this concentration increased (Fig. 3). When only DNA was added to the cells no hemolysis occurred, suggesting that the amphiphile is pertinent to hemolysis. Indeed, as shown in Fig. 3B (open circle), the amphiphile per se triggers hemolysis while at low amphiphile concentrations complexation with a fixed amount of DNA apparently prevents the amphiphile from perforating the cell membrane. When the amphiphile concentration is further increased, the effect of amphiphile-induced leakage becomes more prominent, consistent with the data in Fig. 3A, although a synergistic effect of DNA appears apparent in this case (Fig. 3B). In Fig. 3C the results are given of the hemolysis of erythrocytes induced by a complex of pCAT of 4.7 kb with vesicles consisting either of DOTMA (open symbols) or DOTMA/DOPE (filled symbols). As shown, hemolysis was most efficient when the vesicles also contained DOPE. Note that the effective concentration of DOTMA in the pure system is twice that used in the mixed formulation (DOTMA/DOPE).

In summary, the foregoing experiments indicate that a distinct amount of amphiphile is critical, not only with respect to transfection efficiency, but also in its ability to govern hemolytic properties of the DNA/amphiphile complex. Therefore, it was of interest to further characterize the nature of the complex.

3.3. DNA–amphiphile interaction

In order to shed more light on the nature and structural consequences of binding of DNA to the vesicles, studies were performed in which ethidium bromide (EtBr) was used as a probe to report changes in the nucleic acid [17]. It is known that EtBr can intercalate with the base pairs of the DNA. Upon DNA/amphiphile complex formation, the fluorescence intensity will decrease because the EtBr can no longer intercalate between the base pairs of the DNA [18]. As shown in Fig. 4, upon increasing the amount of lipid, the fluorescence intensity decreases, irrespective of the initial size of the vesicles (SUV vs. LUV). However, this decrease is dependent on the length of the DNA

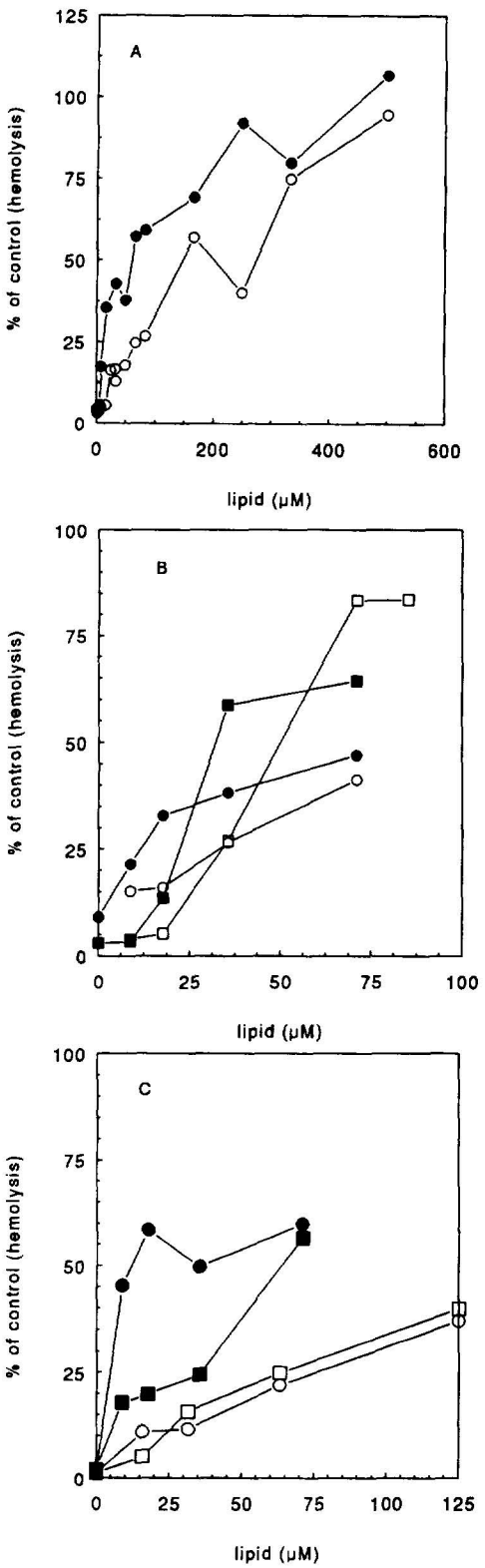

Fig. 3. Amphiphile-induced hemolysis: effect of vesicle/DNA ratio, and DNA length. The 100% value was obtained by treating the cells with 1% Triton X-100, which results in complete cell lysis. (A) Cells were treated either with a complex of DOTMA/DOPE and 1 μg DNA of 4.7 kb (filled circle) or with a complex of DOTMA/DOPE with DNA of 1 μg 18.8 kb (open circle). (B) Vesicles of DOTMA/DOPE were complexed with different amounts of pCAT of 18.8 kb: 0 μg (open circle), 1 μg (filled circle), 5 μg (filled square) or 10 μg (open square). (C) Vesicles of DOTMA/DOPE (filled symbols) or vesicles of only DOTMA (open symbols) were complexed with different amounts of pCAT of 4.7 kb: 1 μg (circle) or 5 μg (square).

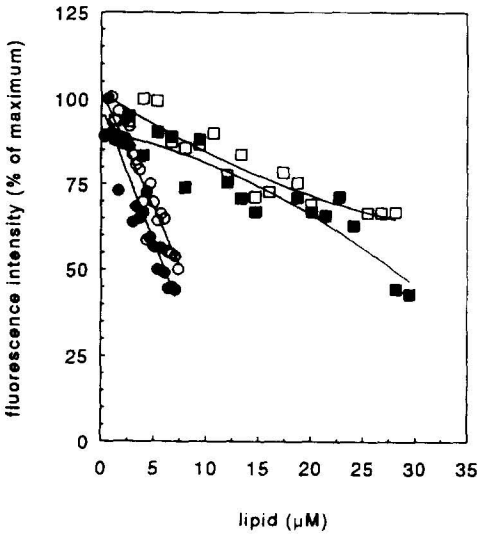

Fig. 4. Effect of vesicle/DNA ratio on the EtBr-fluorescence intensity. EtBr was added to DNA (10 μg) and DNA, complexed to DOTMA/DOPE vesicles, at a final concentration of 0.2 μg/ml. The length of the DNA was varied as well as the size of the vesicles. SUV + 10 μg DNA of 4.7 kb (filled circle). SUV + 10 μg DNA of 18.8 kb (filled square). LUV + 10 μg DNA of 4.7 kb (open circle) and LUV + 10 μg DNA of 18.8 kb (open square). The fluorescence was measured as described in Section 2.

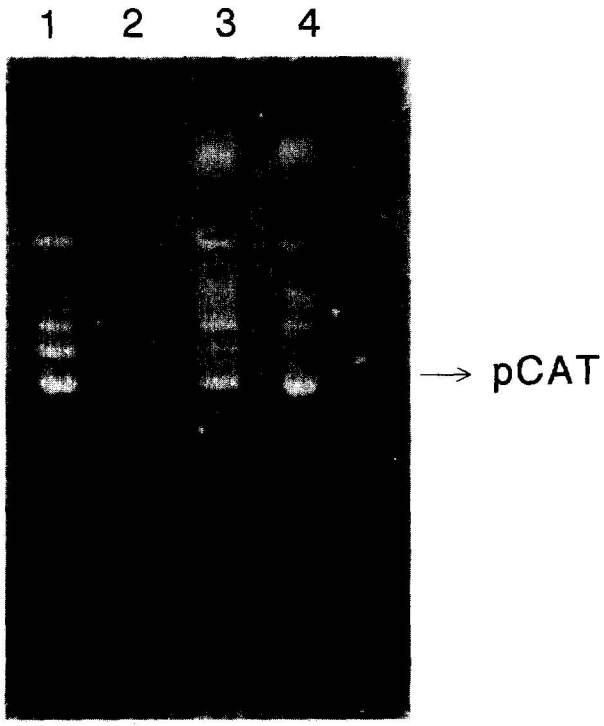

Fig. 5. Effect of DNase I on the degradation of 1 μg pCAT complexed with 36 μM of DOTMA/DOPE vesicles. The complex was incubated for 10 minutes with 10 units of DNase I and, when indicated, treated with Triton X-100 (final concentration, 1% v/v). Lane 1: pCAT; lane 2: pCAT treated with DNase I; lane 3: pCAT complexed to DOTMA/DOPE vesicles, after treatment with DNase I, followed by Triton X-100, lane 4: pCAT complexed to DOTMA/DOPE vesicles, treated with Triton X-100.

strand: the shorter the DNA the steeper the decrease in fluorescence intensity. A further decrease in fluorescence could not be observed. Adding EtBr prior to or after complex formation had no influence on the fluorescence intensity.

Capaccioli et al. [19] have shown that DOTMA is able to prevent oligodeoxynucleotides from degradation in cultured cells and in serum. To determine whether the plasmid DNA used in the present work was similarly protected by the vesicles, the complex was treated with DNase and subsequently applied on an agarose gel.

The untreated material shows a clear DNA band, representing pCAT (Fig. 5, lane 1). After an incubation with 10 units of DNase I for 15 min at 37°C, DNA is no longer detectable on the gel due to the complete degradation of DNA (Fig. 5, lane 2). However, an effective protection to degradation was provided, as revealed by the presence of a clear DNA band (Fig. 5, lane 3), when the DNA had been complexed to the vesicles, prior to treatment with DNAse I. Note that the DNA band is located at the same height as that of untreated, control pCAT and pCAT, dissociated from the complex, upon treatment with Triton X-100 (Fig. 5, lanes 1 and 4).

It has been shown that the positive charges on the DOTMA molecules are important for transfection [9]. In order to determine the extent to which charge is involved in mediating transfection, cells were treated with $CaCl_2$ or with EGTA just prior to the incubation with the vesicle/DNA complex. When the cells were washed with $CaCl_2$, transfection is inhibited by almost 85%, while transfection was not effected when washing the cells with EGTA (Fig. 6). The inhibition caused by $CaCl_2$ could not be reversed by treating the cells with EGTA after the

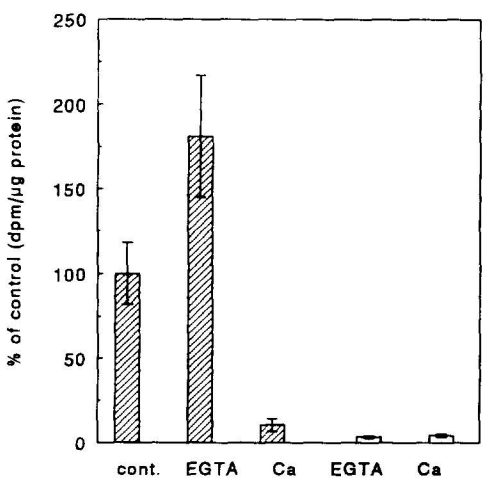

Fig. 6. Effect of calcium pretreatment on the transfection of COS-7 cells. COS-7 cells were transfected with 1 μg DNA complexed with 71 μM DOTMA/DOPE (1:1) vesicles (hatched bar) or with 1 μg DNA only (open bar). Just prior to initiating transfection by adding the DNA/amphiphile complex, the cells were washed with 10 mM $CaCl_2$ or with 50 mM EGTA. The results are expressed as the mean of three experiments ± S.D.

incubation with $CaCl_2$, suggesting that the inhibitory effect of Ca^{2+} is not due to a simple neutralization of charges. Indeed, some specificity of these Ca^{2+}-sensitive interaction sites was suggested by proteolytic treatment of the cells. Thus, transfection was efficiently abolished when COS-7 cells were preincubated with 0.1% pronase for 1 h at 0°C, prior to starting transfection (results not shown). These results may indicate that (a) cell surface protein(s) play(s) a role in transfection.

4. Discussion

Cationic amphiphiles are now widely used as an effective tool in delivering DNA into mammalian cells [3–6,22]. Yet little is known about the mechanism that governs DNA uptake. Such knowledge would be advantageous in optimizing and extending the carrier properties of amphiphiles with regard to cell type and compounds capable of being introduced via this carrier system. Indeed, the present study and previous work has shown that transfection is dependent on the cell type used [20,21]. It is possible that distinct cell surface proteins play a crucial role in the delivery of the DNA into the cell as proteolytic treatment abolished transfection. These potential proteinaceous transfer sites are then further characterized by a high Ca^{2+} sensitivity (Fig. 6). Hence, the absence of these proteins may explain why certain cell types such as fibroblasts are more difficult or even impossible to transfect. However, it cannot be excluded yet that cell type dependent differences in transfection efficiency are, at least in part, due to a diminished transcription efficiency of the DNA brought into the cell.

The size of the complex seems to be an important parameter as the transfection efficiency decreases when the size of the plasmid increases (Fig. 2B). Moreover since the initial size of the vesicles had no effect on transfection efficiency (Fig. 2A) it is tempting to suggest that the nucleic acid determines the size of the complex by recruiting amphiphile molecules, rather than that the size is determined by association of DNA to amphiphile vesicles as such. The fact that the presence of DNA can inhibit amphiphile-induced hemolysis at a relatively low amphiphile concentration would be entirely consistent with such a notion (Fig. 3). These results are further confirmed by the EtBr intercalation studies (Fig. 4). It is evident that the vesicle size has no influence on the decrease in EtBr-fluorescence intensity, indicating that the encapsulation volume is irrelevant in the 'DNA-packaging' process, whereas the length of the DNA strand does have an influence, in contrast to the results found by Gershon et al. [17].

When the concentration of the amphiphile increases (at a fixed amount of DNA), the transfection efficiency increases, suggesting that a certain exposure of the amphiphile per se is a prerequisite for the complex to interact with the cell surface, a minimal requirement for translocation. The critical nature of this requirement is emphasized by the observation that a strongly reduced transfection is observed when the concentration of amphiphile relative to DNA becomes too high. At such conditions the amphiphile becomes toxic which is reflected by an enhanced degree of hemolysis (Fig. 3). Hence, in line with previous suggestions [4] a critical amphiphile/DNA ratio is crucial for an optimal transfection, dictated by an optimal complex–cell surface interaction, and a minimal toxic effect, which may result from amphiphile-induced membrane permeabilization. On the other hand, permeabilization or pore formation may well be essential for efficient translocation of DNA and/or the amphiphile/DNA complex. In this context it should be noted that an incubation time of several hours is a prerequisite for obtaining significant transfection efficiency. Indeed, a wash of the cells and removal of the complex from the medium after a 2 h incubation abolishes transfection [4], (unpublished observation). The time scale of these events is difficult to reconcile with a fusion-mediated mechanism for the DNA delivery [4,11]. Fusion is expected to take place with much faster kinetics, as shown for the interaction of vesicles composed of pure synthetic amphiphiles and erythrocytes [23] and taking into account the fusogenic properties of the complex per se [24]. Furthermore, when endocytosis would represent a major pathway of entry, a 2 h time interval should suffice for substantial internalization of plasma membrane-bound vesicles, given a membrane surface internalization rate of approx. 50%/h [25]. Hence a pore formation mechanism may serve as an appropriate alternative, providing a rational for geometrical, i.e., size-dependent constraints in DNA delivery as well (Fig. 2). Thus, although hemolysis occurs to the same extent in both cases, only small plasmids efficiently enter the cell, as reflected by transfection efficiency. This could imply that translocation as such across the pore may constitute the rate-limiting step in overall transfection.

An intriguing observation relevant to intracellular delivery of nucleic acids, that remains to be corroborated further involves the potential ability of DNA to act synergistically in bringing about amphiphile-induced permeabilization (Fig. 3B and C), when a critical vesicle to DNA ratio is reached. At a relatively low lipid/DNA ratio, the nucleic acid prevents the amphiphile from perforating the cell membrane. When the amphiphile concentration in the complex increases, the amount of DNA is not sufficient to protect the cell membrane and hemolysis occurs. At still higher amphiphile concentrations, DNA synergism becomes effective. It is thus likely that during the transfection experiments the toxicity, which occurs at higher vesicle concentration, is not only due to the amphiphiles, but also to the synergistic effect of the DNA. In Fig. 3B an S-shaped curve defines the course of the different DNA concentrations investigated. Upon increasing the DNA concentration, the S-curve shifts to the right and the plateau

value increases with increasing DNA concentration. When the vesicle to DNA ratio is calculated for which the synergistic function of the DNA on hemolysis takes over its protective function, it is striking that it occurs at a ratio of 4:1 in all cases. Whether this ratio dictates a particular complex conformation remains to be established. However, it is known that charge neutralization of DNA, which occurs upon interaction with positively charged DOTMA, can transform the circular plasmid structure into a doughnut-like shape. Such a change in morphology may be of relevance for efficient membrane translocation.

As shown in Fig. 3C, hemolysis of erythrocytes induced by vesicles composed of only DOTMA, is much less than hemolysis induced by vesicles composed of DOTMA/DOPE. Moreover, DNA does not display a synergistic effect nor does it prevent hemolysis when complexed to the DOTMA vesicles. It can thus be concluded that the presence of DOPE in the vesicles strongly facilitates the membrane destabilizing properties of the overall complex. This is confirmed by transfection studies that were carried out with vesicles composed of only DOTMA as the carrier system. With this carrier system the transfection efficiencies were less than those obtained with DOTMA/DOPE vesicles as carriers (results not shown).

We conclude that although membrane fusion may play a key role in DNA packaging, i.e., amphiphile/DNA complex formation (Gershon et al. [17]), current evidence may favor translocation via a 'perturbed target-membrane' mechanism rather than by fusion as the mechanism by which nucleic acids are introduced into the cells.

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